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Ascorbic acid and N-acetyl cysteine prevent uncoupling of nitric oxide synthase and increase tolerance to ischemia/reperfusion injury in diabetic rat heart

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Abstract

Oxidative stress may cause a loss of tetrahydrobiopterin (BH4), a co-factor of nitric oxide synthase (NOS), decrease the bioavailability of NO and aggravate ischemia/reperfusion (I/R) injury in diabetic heart. We hypothesized that ascorbic acid (AA) and N-acetyl cysteine (NAC) protect the diabetic heart from I/R injury by increasing BH4/dihydrobiopterin (BH2) ratio and inhibiting uncoupling of NOS. Diabetes mellitus was induced in rats by streptozotocin treatment, and the hearts were isolated and perfused. BH4 and BH4/BH2 ratio decreased in the diabetic heart associated with increased production of superoxide and nitrotyrosine (NT). Treatment with AA or NAC significantly increased BH4/BH2 ratio in the diabetic heart associated with decreased production of superoxide and NT and increased generation of nitrate plus nitrite (NOx). Pre-treatment with AA or NAC before 30 min ischemia followed by 120 min reperfusion improved left ventricular (LV) function and reduced infarct size in the diabetic but not non-diabetic hearts. The NOS inhibitor, L-NAME, inhibited the increase in the generation of superoxide, NT and NOx, but aggravated LV function and increased infarct size in the diabetic heart. L-NAME also abrogated the increase in NOx and improvement of LV function and the infarct size-limiting effect induced by AA or NAC in the diabetic heart. These results suggest that AA and NAC increase BH4/BH2 ratio and prevent NOS uncoupling in the diabetic heart. Resultant increase in the bioavailability of NO renders the diabetic heart toleratant to I/R injury.

Keywords: *nitric oxide synthase, tetrahydrobiopterin, diabetes mellitus, ischemia, reperfusion, cardioprotection*

Introduction

It has been established that the patients with diabetes mellitus (DM) have a poor prognosis after myocardial infarction at least in part due to the susceptibility of the diabetic heart to myocardial ischemia/reperfusion (I/R) injury [1,2]. Although the detrimental effect of DM on the cardiovascular system is multifactorial, oxidative stress has been implicated in the pathogenesis of enhanced myocardial I/R injury in the diabetic heart [3,4].

Oxidative stress in DM is known to deplete tetrahydrobiopterin (BH4) by oxidation to dihydrobiopterin (BH2) [5,6]. BH4 is a co-factor of the three major isoforms of NOS, endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS, and is necessary for NO biosynthesis. The coupling status of NOS is determined by the BH4/BH2 ratio [7]. Under the normal condition where BH4 is abundant and binds the dimer interface of NOS, electron flow through NADPH, FAD, FMN and calmodulin complex generates NO from L-arginine and oxygen [8,9]. However, the loss of BH4 and resultant decrease in BH4/BH2 ratio replace BH4 in the dimer interface of NOS with BH2 and cause uncoupling of NOS, leading

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to the generation of more superoxide and less NO. Thus, NOS uncoupling aggravates oxidative stress and reduces bioavailable NO, which may inhibit cardioprotective signal transduction against I/R injury [10]. Indeed, reduced BH4 level and uncoupling of NOS have been implicated in the pathophysiology of I/R injury [11].

iNOS is up-regulated in the diabetic hearts by oxidative stress, and uncoupling of iNOS may play a more crucial role in the pathophysiology of cardiovascular function than uncoupling of eNOS in these hearts [12]. We have previously shown that iNOS uncoupling plays a predominant role in oxidative/nitrosative stress in the diabetic heart, and that reversal of iNOS uncoupling by treatment with BH4 unmasks tolerance to I/R injury by increasing the bioavailability of iNOS-derived NO [13]. Thus, increasing BH4 by supplying BH4 or eliminating oxidative stress in the diabetic heart may represent a promising approach to prevent I/R injury.

In contrast to an increase in the hydrophobic antioxidant, vitamin E, the hydrophilic antioxidants, ascorbic acid (AA) and glutathione, are depleted in the diabetic heart [14–17]. The glutathione precursor, N-acetyl-L-cysteine (NAC), has been shown to protect from hyperglycemia-induced myocyte cell death by scavenging reactive oxygen species and replenishment of intracellular glutathione content [18]. The improvement of cellular redox state by AA and NAC could increase BH4 and BH4/BH2 ratio, normalize coupling status of NOS, increase the bioavailability of NO, thereby presumably conferring myocardial protection. However, no studies have been undertaken to investigate the effect of AA and NAC on the coupling status of NOS and myocardial protection. The present study was designed to address causal relationship between reversal of NOS uncoupling and cardioprotection by AA and NAC in the diabetic heart. To avoid confounding effects of hyperglycemia on cardiovascular function *in vivo*, we used an isolated and buffer-perfused rat heart preparation.

Material and methods

Animal preparation

Male Sprague-Dawley rats weighing 250–300 g were used in the present study. All animals were handled in accordance with the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of the Care and Use of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised in 1996. The study was approved by the Animal Care Committee of Kansai Medical University (Moriguchi, Japan).

Rats were injected with 0.5 ml of phosphate-buffered saline (PBS) or streptozotocin (STZ; Sigma Chemical Co., St. Louis, MO, USA) at a dose of 50 mg/kg/day into the intraperitoneal space for 5 days to create DM. Blood glucose was measured by using Medisafe-Mini glucose test strips read on a glucometer (Terumo, Tokyo, Japan). All the rats used in the present study showed blood glucose level $>$ 350 mg/dl 28 days after STZ treatment.

Experimental protocol and perfusion technique

Experimental protocol is shown in Figure 1 (Figure 1). Twenty-eight days after STZ treatment, the rats were anaesthetized intraperitoneally with pentobarbital sodium (100 mg/kg). The heart was isolated and perfused at a constant mean pressure of 70–75 mmHg using a Krebs-Henseleit bicarbonate (KHB) buffer solution of the following composition (in mM): 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.8 $CaCl_2$ and 11 glucose; pH 7.4 at 37°C when equilibrated with a mixture of 95% O_2–5% CO_2 gas. Coronary flow (CF) and isovolumic left ventricular (LV) function were measured as described previously [19]. A latex balloon was inserted into the LV through the left atrium and filled with saline to produce an LV end-diastolic pressure (LVEDP) of 5–10 mmHg at baseline. The balloon volume was kept constant throughout the experiment. CF and LV developed pressure (LVDP) were expressed as percent of baseline, because baseline CF and LVDP were significantly lower at comparable perfusion pressure and LVEDP in the diabetic heart compared to the control heart.

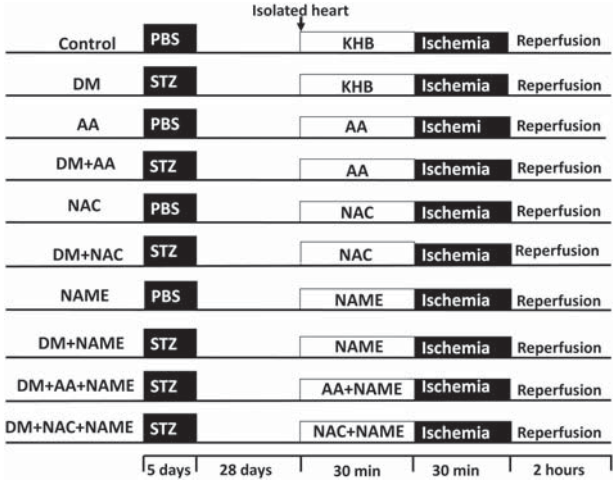

Figure 1. Experimental protocol. Rats were injected with phosphate-buffered saline (PBS) or streptozotocin (STZ) into the intraperitoneal space for 5 days to induce diabetes mellitus (DM). Twenty-eight days later, the rats were sacrificed and the hearts were isolated and perfused with Krebs-Henseleit bicarbonate (KHB) buffer solution for 30 min. Then, the hearts were treated with ascorbic acid (AA), N-acetyl cysteine (NAC) or N-ω-nitro-L-arginine methyl ester (NAME) in the presence or absence of AA or NAC for 30 min followed by 30 min ischemia and 2 h reperfusion.

After measurements of the baseline data, the hearts were randomly treated with 1 mM AA or 0.1 mM NAC (both obtained from Sigma) for 30 min. The concentrations of AA and NAC were determined according to the studies performed to elicit the optimal effect of these antioxidants [20,21]. To elucidate the role of NOS, N-ω-nitro-L-arginine methyl ester (L-NAME) at a dose of 0.1 mM, which sufficiently inhibited NOS-mediated cardioprotection in the isolated and perfused rat heart [22], was administered for 30 min. These hearts were then subjected to 30 min of global ischemia, followed by 120 min of reperfusion. LV function was measured at baseline, just prior to ischemia and 30 min after reperfusion, because the recovery of LV function after reperfusion reached a maximum within 30 min.

Measurements of myocardial GSH and GSSG

Thirty minutes after perfusion on the Langendorff apparatus, the heart was rapidly excised and snap-frozen in liquid nitrogen. Frozen myocardial tissue samples were homogenized (g/10 ml) in 5% metaphosphoric acid. Myocardial GSH/GSSG ratio was measured using a Bioxytech GSH/GSSG-412 colorimetric assay kit from Oxis Research (Portland, OR, USA) as described previously [23].

Measurement of myocardial biopterin concentrations

BH4 and BH2 were measured in cardiac homogenates by high performance liquid chromatography analysis after iodine oxidation in acidic or alkaline conditions as described previously [24].

Measurement of superoxide production

Superoxide production was assessed *in vivo* using dihydroethidium (DHE) as described previously [13]. DHE is a cell-permeable fluorescent dye that is oxidized by superoxide to fluorescent ethidium bromide. Ethidium bromide is a nucleic acid stain, trapped by intercalation with DNA. The fluorescence intensity of DHE indicates the relative levels of superoxide production. DHE (prepared in DMSO as a 2mM stock solution) was added to the KHB buffer (final concentration 5μM), and the hearts were perfused for 30min at 37°C. Frozen sections were prepared as described before, and cardiomyocytes were immunostained with α-actinin (Sigma) as a primary antibody and a FITC-conjugated secondary antibody. Images were obtained with a confocal laser microscope (Fluo View, Olympus, Tokyo, Japan) and fluorescence was detected with a 585-nm long-pass filter. Background was identified as an area without cells. In each heart, 100 nuclei were randomly selected by an observer blind to the identity of the samples, and the pixel intensity in each nucleus was analyzed by subtraction of background fluorescence. The mean fluorescence intensity of the nuclei was then determined using the image analyzing software system Win Roof (Mitani, Fukui, Japan).

Measurement of nitrotyrosine formation

Nitrotyrosine (NT) formation in the heart was measured by an ELISA method. Heart tissue (100 mg), sampled from the frozen and subsequently powdered left ventricle, was homogenized in 200 μl of RIPA buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS. The samples were centrifuged at 10,000 **g** at 4°C for 10 min. A 50 μl aliquot of supernatant was removed and 3-NT was quantified using an NT ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instruction.

Measurement of NOx generation

Nitrite and nitrate (NOx), stable oxidation metabolites of NO, have been measured as an index for bioavailability of NO [25]. Myocardial level of NOx was measured by a HPLC method. Heart tissue (100 mg), sampled from the frozen and subsequently powdered left ventricle, was homogenized in 500 μl of extraction buffer containing 50 mM Tris (pH 7.4), 1 mM DTT and 1 mM EDTA. The samples were centrifuged at 10,000 **g** at 4°C for 10 min. A 300 μl aliquot of supernatant was removed, and NOx was measured using a HPLC system (Shimadzu Co. Kyoto, Japan) according to the method as described by Green at al. [26].

Measurement of infarct size

Infarct size was measured 120 min after reperfusion. Infarct size measurements were performed by a triphenyltetrazolium chloride (TTC) (Sigma) staining method as described previously [19]. Infarct size was expressed as percent of LV mass.

Statistical analysis

Statistical analysis was conducted with a commercially available software package (StatView 5.0, SAS Institute Inc, Cary, NC, USA). Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. Two-way repeated-measures ANOVA followed by Fisher's PLSD test was applied to compare serial measurements of variables. All numerical data are expressed as mean ± SE. The differences were considered significant at a p value of <0.05.

Results

GSH/GSSG ratio

GSH/GSSG ratio significantly decreased in the diabetic heart (Figure 2). Only NAC significantly increased GSH/GSSG ratio in the control heart. However, either AA or NAC normalized GSH/GSSG ratio in the diabetic heart. L-NAME had no significant effect on GSH/GSSG ratio in the control and diabetic hearts. L-NAME also did not affect AA- and NAC-induced increase in GSH/GSSG ratio in the diabetic heart.

BH4 and BH2

BH4 content significantly decreased in the diabetic hearts, while BH2 content significantly increased in these hearts (Figure 3A), resulting in a marked decrease in BH4/BH2 ratio (Figure 3B). Neither AA nor NAC affected BH4 and BH2 content and BH4/BH2 ratio in the control heart. However, either AA or NAC significantly increased BH4/BH2 ratio by decreasing BH2 content without significantly increasing BH4 content in the diabetic heart. L-NAME had no significant effect on BH4 and BH2 content in the control and diabetic heart nor did it affect AA- or NAC-induced increase in BH4/BH2 ratio.

Superoxide production

Confocal laser microscopy showed that DHE was accumulated in the nuclei in cardiomyocytes and non-cardiomyocytes in the diabetic heart (Figure 4A). This accumulation of DHE was diminished by treatment with AA, NAC or L-NAME. Quantitative analysis demonstrated that DHE accumulation in the nuclei was markedly increased in the diabetic heart compared to the control heart (Figure 4B). AA, NAC or L-NAME significantly inhibited the DHE accumulation. L-NAME did not affect AA- and NAC-induced inhibition of DHE accumulation in the diabetic heart.

Nitrotyrosine formation

3-NT was significantly increased in the diabetic heart (Figure 5). The increase in NT formation in the diabetic heart was significantly inhibited by treatment with AA, NAC or L-NAME. L-NAME did not affect AA- and NAC-induced inhibition of 3-NT formation in the diabetic heart.

NOx generation

NOx generation was significantly increased in the diabetic heart (Figure 6). AA and NAC had no effect on NOx generation in the control heart but significantly augmented NOx generation in the diabetic heart. This increase in NOx generation in the diabetic heart was abolished by L-NAME. L-NAME also significantly attenuated AA- or NAC-induced augmentation of NOx generation in the diabetic heart.

LV function

AA and NAC significantly increased CF and LVDP in the diabetic heart before ischemia (Table 1). L-NAME had no significant effect on CF, LVDP and LVEDP in the control heart. However, L-NAME significantly decreased CF, LVDP and heart rate (HR), and increased LVEDP in the diabetic heart before ischemia. L-NAME also abolished the AA

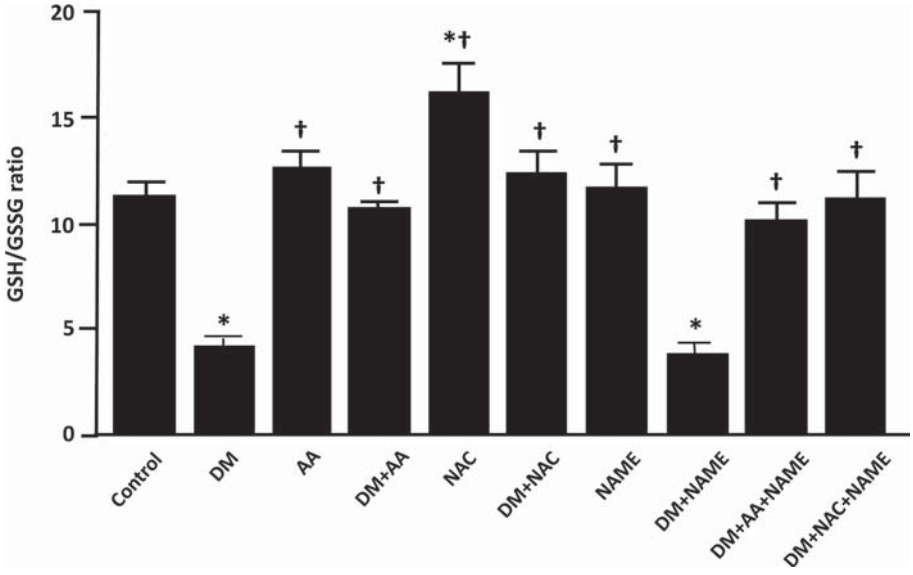

Figure 2. GSH/GSSG ratio. Each bar graph indicates mean ± SEM of five experiments. $^{*}p < 0.05$ vs control, $^{\dagger}p < 0.05$ vs DM. DM, diabetes mellitus; AA, ascorbic acid; NAC, N-acetyl cysteine; NAME, N-ω-nitro-L-arginine methyl ester.

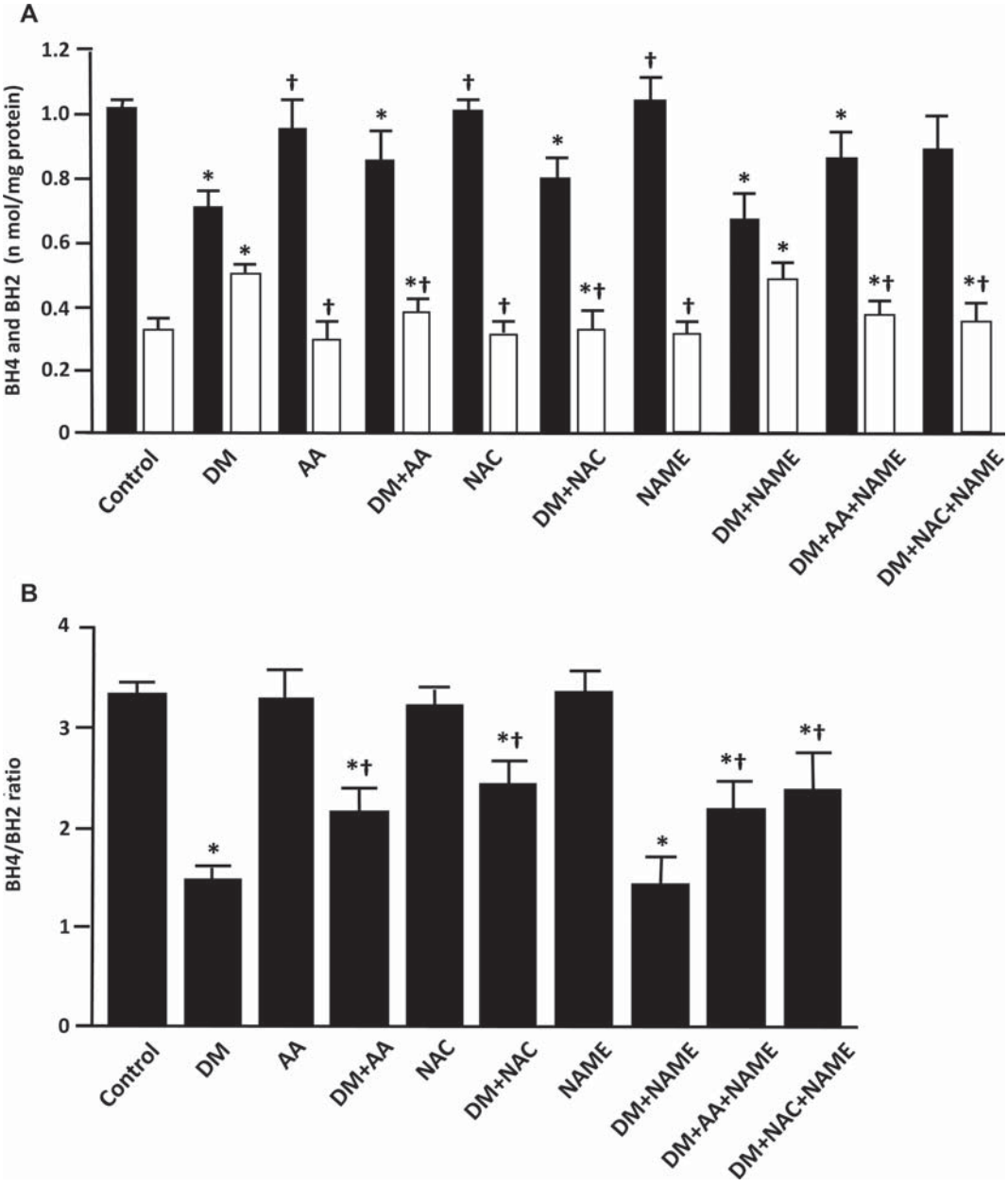

Figure 3. (A) Myocardial BH4 and BH2 content. Closed bars and open bars indicate BH4 and BH2, respectively. Each bar graph indicates mean ± SEM of five experiments. $^{*}p < 0.05$ vs control, $^{\dagger}p < 0.05$ vs DM. DM, diabetes mellitus; AA, ascorbic acid; NAC, N-acetyl cysteine; NAME, N-ω-nitro-L-arginine methyl ester. (B) BH4/BH2 ratio. Each bar graph indicates mean ± SEM of 5 experiments. $^{*}p < 0.05$ vs control, $^{\dagger}p < 0.05$ vs DM. DM, diabetes mellitus; AA, ascorbic acid; NAC, N-acetyl cysteine; NAME, N-ω-nitro-L-arginine methyl ester.

or NAC-induced increase in CF and LVDP in the diabetic heart before ischemia. After I/R, CF, HR and LVDP significantly decreased and LVEDP significantly increased in all groups of hearts. There was no significant difference in these functional parameters between the control and diabetic hearts during reperfusion. Pre-treatment with AA and NAC had no significant effect on the recovery of CF and LV function in the control heart. However, these antioxidants significantly improved CF and LV function in the diabetic heart. L-NAME had no significant effect on CF and LV function in the control heart. However, L-NAME significantly deteriorated CF and LV function in the diabetic heart and abrogated the improvement of CF and LV function conferred by AA and NAC in the diabetic heart.

Infarct size

Infarct size was not significantly different between the control and diabetic hearts (Figure 7). Although AA and NAC did not reduce infarct size in the control heart, these antioxidants significantly decreased infarct size in the diabetic heart. L-NAME did not affect infarct size in the control heart, but significantly increased infarct size in the diabetic

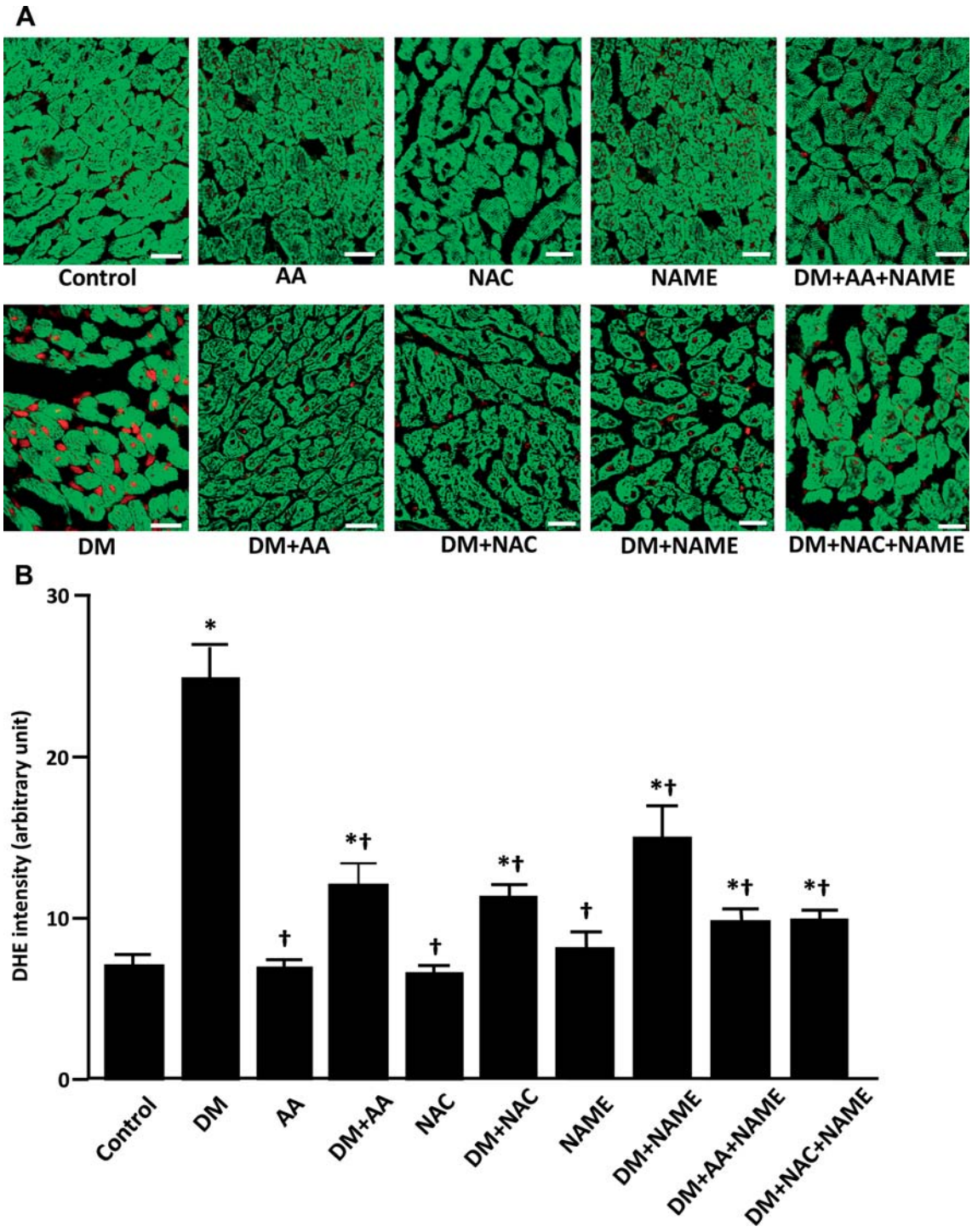

Figure 4. (A) Representative immunohistochemical images of dihydroethidium (DHE) staining. DHE-accumulated nuclei are stained red and cardiomyocytes are stained green. Bars indicate 20 μm. DM, diabetes mellitus; AA, ascorbic acid; NAC, N-acetyl cysteine; NAME, N-ω-nitro-L-arginine methyl ester. (B). Quantitative analysis of DHE staining. Each bar graph indicates mean ± SEM of five experiments. $^{*}p < 0.05$ vs control, $^{\dagger}p < 0.05$ vs DM. DM, diabetes mellitus; AA, ascorbic acid; NAC, N-acetyl cysteine; NAME, N-ω-nitro-L-arginine methyl ester.

heart. L-NAME also abrogated the infarct size-limiting effect of AA and NAC in the diabetic heart.

Discussion

The hydrophilic antioxidants, AA and glutathione, are depleted in the diabetic heart [15–17]. Such a loss of AA and glutathione may underlie the vulnerability of the diabetic heart to I/R injury at least in part due to the increase in oxidative stress [3,4]. Oxidative stress in turn causes uncoupling of NOS by depleting BH4, leading to further oxidative stress and loss of the bioavailability of NO. Therefore, replenishment of these antioxidants was thought to be a

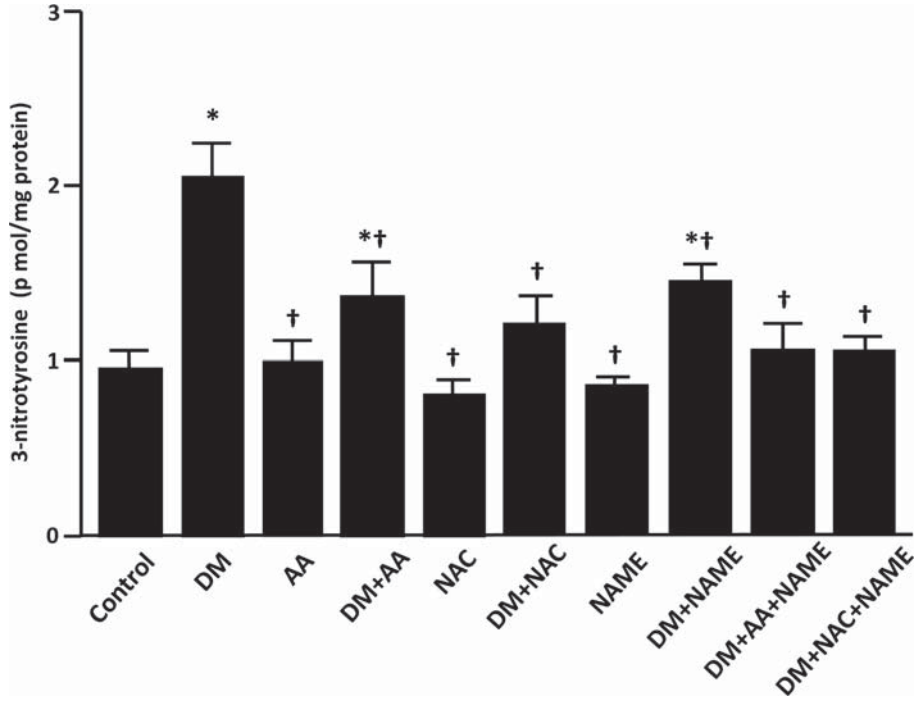

Figure 5. 3-nitrotyrosine formation. Each bar graph indicates mean ± SEM of five experiments. $^{*}p < 0.05$ vs control, $^{\dagger}p < 0.05$ vs DM. DM, diabetes mellitus; AA, ascorbic acid; NAC, N-acetyl cysteine; NAME, N-ω-nitro-L-arginine methyl ester.

promising approach to increase the tolerance to I/R injury in the diabetic heart. The present study demonstrated that AA and NAC increased GSH/GSSG ratio in the diabetic heart associated with an increase in BH4/BH2 ratio, which is a primary determinant of the coupling status of NO [7]. The increase in BH4/BH2 ratio was more attributed to a decrease in BH2 than an increase in BH4. Because it has been demonstrated that the *de novo* pathway for BH4 synthesis is inactivated in the diabetic heart [27,28], our finding suggests that although BH4 synthesis was reduced in the diabetic heart, AA or NAC prevented oxidation of BH4 to BH2. These antioxidants decreased superoxide production and NT formation and increased the generation of NO. This increase in NO generation was abolished by co-administration with L-NAME, indicating that AA and NAC inhibited uncoupling of NOS in the diabetic heart.

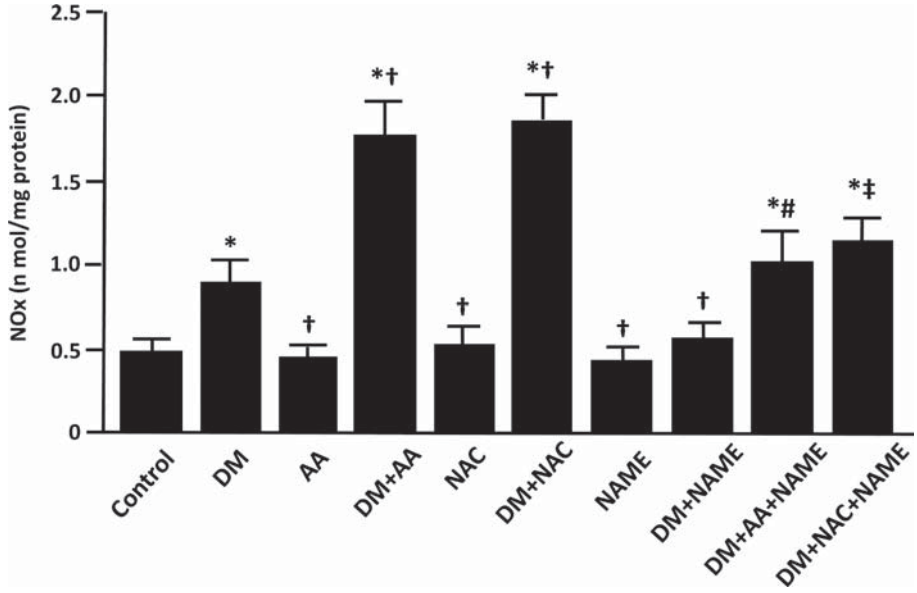

Figure 6. Nitrite and nitrate (NOx) generation. Each bar graph indicates mean ± SEM of five experiments. $^{*}p < 0.05$ vs control, $^{\dagger}p < 0.05$ vs DM, $^{\#}p < 0.05$ vs DM + AA, $^{\ddagger}p < 0.05$ vs DM + NAC. DM, diabetes mellitus; AA, ascorbic acid; NAC, N-acetyl cysteine; NAME, N-ω-nitro-L-arginine methyl ester.

Table I. Effect of ascorbic acid and N-acetyl-L-cysteine on CF and LV function.

	Baseline	PI	30 min R
CF (% baseline)			
Control (n = 5)	100	100 ± 2	47 ± 2*
DM (n = 5)	100	102 ± 1	48 ± 2*
AA (n = 5)	100	100 ± 1	51 ± 3*
DM + AA (n = 5)	100	114 ± 2*†#	62 ± 3*†#
NAC (n = 5)	100	101 ± 1	50 ± 3*
DM + NAC (n = 5)	100	116 ± 2*†#	65 ± 3*†#
NAME (n = 5)	100	98 ± 1	45 ± 3*
DM + NAME (n = 5)	100	91 ± 2*†#	37 ± 2*†#
DM + AA + NAME (n = 5)	100	98 ± 1‡	46 ± 2*‡
DM + NAC + NAME (n = 5)	100	99 ± 2¶	46 ± 2*¶
HR (rpm)			
Control (n = 5)	318 ± 9	317 ± 9	229 ± 16*
DM (n = 5)	316 ± 11	318 ± 9	237 ± 11*
AA (n = 5)	312 ± 10	310 ± 9	241 ± 13*
DM + AA (n = 5)	322 ± 7	332 ± 8	278 ± 11*†#
NAC (n = 5)	315 ± 6	317 ± 7	229 ± 16*
DM + NAC (n = 5)	311 ± 8	329 ± 7	277 ± 11*†#
NAME (n = 5)	318 ± 9	315 ± 7	229 ± 17*
DM + NAME (n = 5)	310 ± 6	279 ± 5*†#	180 ± 8*†#
DM + AA + NAME (n = 5)	318 ± 8	312 ± 5	227 ± 16*‡
DM + NAC + NAME (n = 5)	319 ± 9	317 ± 6	231 ± 12*¶
LVDP (% baseline)			
Control (n = 5)	100	100 ± 1	46 ± 4*
DM (n = 5)	100	100 ± 2	47 ± 4*
AA (n = 5)	100	100 ± 2	48 ± 2*
DM + AA (n = 5)	100	106 ± 2*†#	62 ± 3*†#
NAC (n = 5)	100	101 ± 2	51 ± 2*
DM + NAC (n = 5)	100	105 ± 1*†#	67 ± 4*†#
NAME (n = 5)	100	100 ± 1	43 ± 5*
DM + NAME (n = 5)	100	93 ± 1*†#	34 ± 4*†#
DM + AA + NAME (n = 5)	100	99 ± 1	49 ± 4*‡
DM + NAC + NAME (n = 5)	100	100 ± 1	52 ± 4*¶
LDEDP (mmHg)			
Control (n = 5)	7.8 ± 0.6	7.8 ± 1.0	41 ± 3.6*
DM (n = 5)	7.2 ± 0.9	7.5 ± 0.9	38 ± 3.6*
AA (n = 5)	7.4 ± 0.7	7.2 ± 0.6	39 ± 4.5*
DM + AA (n = 5)	8.4 ± 0.8	8.1 ± 0.8	27 ± 3.7*†#
NAC (n = 5)	7.9 ± 0.7	7.7 ± 0.8	37 ± 3.4*
DM + NAC (n = 5)	7.5 ± 0.8	7.4 ± 0.7	26 ± 3.9*†#
NAME (n = 5)	7.4 ± 0.7	7.6 ± 0.7	43 ± 4.8
DM + NAME (n = 5)	6.6 ± 0.8	12.3 ± 1.3*†#	56 ± 3.8*†#
DM + AA + NAME (n = 5)	7.0 ± 0.5	7.0 ± 0.5	40 ± 2.7*‡
DM + NAC + NAME (n = 5)	7.7 ± 0.7	7.7 ± 0.7	46 ± 5.8*¶

PI, pre-ischemia; R, reperfusion; CF, coronary flow; HR, heart rate ; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; DM, diabetes mellitus; AA, ascorbic acid; NAC, N-acetyl-L-cysteine; NAME, N-ω-nitro-L-arginine methyl ester. Data are expressed as mean ± SEM. *$p < 0.05$ compared to baseline, †$p < 0.05$ compared to control, #$p < 0.05$ compared to DM, ‡$p < 0.05$ compared to DM + AA, ¶$p < 0.05$ compared to DM + NAC.

The present study demonstrated that the bioavailability of NO was increased in the diabetic heart despite uncoupling of NOS. This finding suggests that net NOS activity is increased in the diabetic heart. The isoform of NOS responsible for enhanced NO generation in the diabetic heart was not investigated in the present study. However, it has been demonstrated that iNOS is up-regulated in the diabetic hearts by oxidative stress, and uncoupling of iNOS may play a more crucial role in the pathophysiology of cardiovascular function than uncoupling of eNOS in these hearts [12,13]. Our recent study indeed demonstrated that iNOS plays a predominant role in oxidative/nitrosative stress compared to eNOS or nNOS in the diabetic heart [29]. However, the role of iNOS should be examined using iNOS selective inhibitors, because L-NAME is more inhibitory to eNOS than iNOS [30]. Nevertheless, the present study suggests that AA or NAC converts NOS from a detrimental to beneficial player by inhibiting uncoupling in the diabetic heart subjected to I/R.

The present study demonstrated that there was no significant difference in LV function and infarct

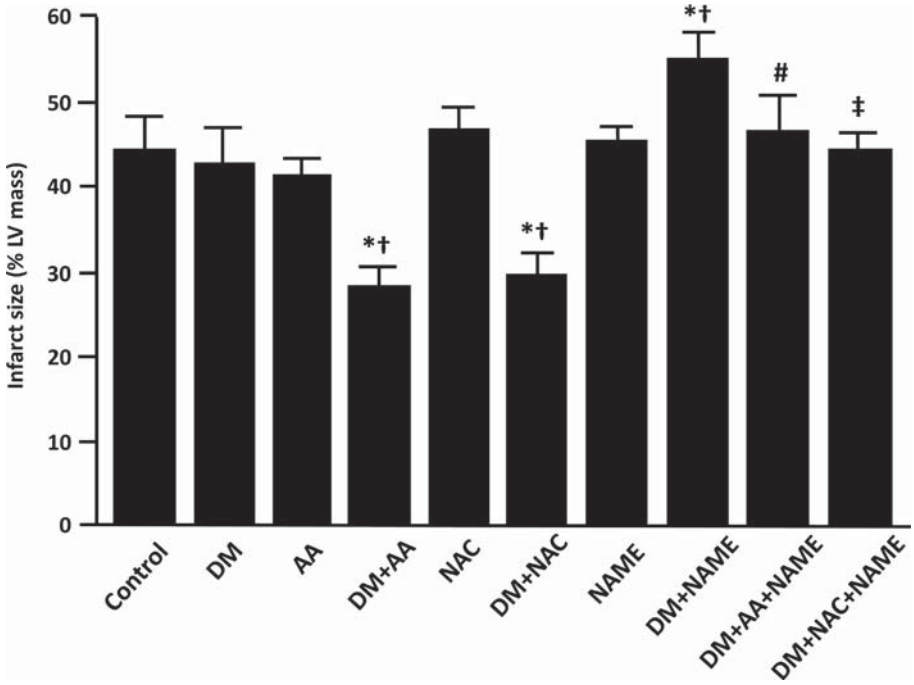

Figure 7. Infarct size. Each bar graph indicates mean ± SEM of five experiments. $^{*}p < 0.05$ vs control, $^{\dagger}p < 0.05$ vs DM, $^{\#}p < 0.05$ vs DM + AA, $^{\ddagger}p < 0.05$ vs DM + NAC. DM, diabetes mellitus; AA, ascorbic acid; NAC, N-acetyl cysteine; NAME, N-ω-nitro-L-arginine methyl ester.

size between the non-diabetic and diabetic hearts. This observation does not necessarily indicate that the diabetic heart is toleratant to I/R injury. L-NAME decreased the production of superoxide and the bioavailability of NO and aggravated LV dysfunction during reperfusion and increased infarct size in the diabetic heart. These findings suggest that increased NO generation but not decreased superoxide production is causally related to AA- or NAC-induced cardioprotection in the diabetic heart, and the diabetic heart becomes susceptible to I/R injury when the bioavailability of NO is reduced by inhibiting NOS, presumably iNOS. In addition, the fact that the cardioprotective effect of AA and NAC in the diabetic heart was abrogated by co-treatment with L-NAME further supports a notion that NO is involved in AA- and NAC-induced cardioprotection in the diabetic heart.

In contrast to significant myocardial protection by treatment with AA or NAC in the diabetic heart, AA and NAC had no significant cardioprotective effect on the control heart. The efficacy of these antioxidants especially with respect to AA in preventing myocardial I/R injury in normal hearts has been a controversial issue. The rats fed a diet containing AA did not show any beneficial effects on the recovery of cardiac function and myocardial damage despite an increased level of myocardial AA content [31]. Moreover, AA did not significantly attenuate post-ischemic injury in the isolated heart of the intact and hypertensive rats [20,32]. By contrast, it has been demonstrated that NAC inhibits oxidative stress and reduces myocardial damage in a dose-dependent manner in the isolated rabbit heart [33]. On the other hand, NAC blocked the cardioprotective effect of ischemic pre-conditioning and post-conditioning [34,35], which is mediated by a redox-sensitive mechanism [36]. Therefore, the effect of AA and NAC on myocardial protection against I/R injury may be dependent on the nitroso-redox balance, which is determined by NOS activity and the magnitude of oxidative stress. The present study points to the conclusion that AA and NAC could elicit a cardioprotective effect by increasing the bioavailability of NO when the heart is subjected to oxidative stress, and NOS is activated but uncoupled by the decrease in BH4/BH2 ratio.

The mechanism by which increased bioavailability of NO by AA and NAC elicits cardioprotection is elusive. There are at least three potential signalling pathways involved in NO-mediated cardioprotection. The first mechanism is protein nitration, which is caused by reaction of protein tyrosine residues with peroxynitrite. Nitration of protein tyrosine residues has been shown to alter the functions of a variety of proteins under physiological and pathophysiological conditions both *in vitro* and *in vivo* [37,38]. Post-translational modification of tyrosine residues has been shown to play an important role in modulating the activity of several PKC isozymes including PKC-ε [39], which has consistently been implicated in the cardioprotective signal transduction [40,41].

However, the fact that inhibition of NT formation by AA and NAC provoked cardioprotection in the diabetic heart may argue against the role of protein nitration in cardioprotective signal transduction in the diabetic heart. The second mechanism is activation of the NO-dependent guanylyl cyclase/cGMP (GC/cGMP) pathway. The cardioprotective action of cGMP has been confirmed by cGMP phosphodiesterase-5 inhibitors in a variety of animal models [42]. However, our previous study [13] demonstrating that a selective GC inhibitor ODQ had no effect on the improvement of CF and LV function and the infarct size-limiting effect of BH4 in the diabetic heart suggests that cGMP may not be a predominant mediator of NO-dependent cardioprotection in the diabetic heart. The third mechanism is protein *S*-nitrosylation. NO can directly modify sulfhydryl residues of proteins through *S*-nitrosylation, which has emerged as an important post-translational protein modification that modulates protein activity [43–45]. Furthermore, *S*-nitrosylation of critical protein thiols has been shown to protect them from further oxidative modification by reactive oxygen species [44,46,47]. This mechanism may be important to protect mitochondria from oxidative stress and Ca^{2+} overload and resultant permeability transition pore opening, which triggers cell death via apoptosis and necrosis [48]. It has become apparent that a number of proteins that participate in cardioprotection undergo *S*-nitrosylation [49]. Our previous study demonstrated that protein *S*-nitrosylation was increased in the diabetic heart, and it was enhanced by BH4 [13]. The fact that a thiol reducing agent, dithiothreitol, inhibited protein *S*-nitrosylation and cardioprotection afforded by BH4 suggests that *S*-nitrosylation may be a principal mechanism for NO-mediated cardioprotection in the diabetic heart. Further studies are warranted to elucidate the mechanism involved in NO-mediated cardioprotection against I/R injury.

In conclusion, the present study demonstrated that AA and NAC increased BH4 and prevented NOS uncoupling in the isolated and perfused diabetic rat heart. The resultant increase in the bioavailability of NO was associated with improvement of post-ischemic LV function and reduction of infarct size. Therefore, AA and NAC may be promising pharmacological tools in protecting the diabetic heart from I/R injury.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This study was supported in part by a Research Grant (No.20590847) from the Ministry of Education, Science, and Culture of Japan.

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